Myeloperoxidase Chlorination and Peroxidation Inhibitor Screening Assay Kit

Item No. 702930

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity	Storage
700164	MPO Assay Buffer (1X)	1 vial/50 ml	4°C
700166	MPO Enzyme (human)	1 vial/40 µl	-20°C
700167	MPO Inhibitor	1 vial/50 µl	4°C
700168	MPO Hydrogen Peroxide	1 vial/100 µl	4°C
400823	MPO Chlorination Detector (NBD-TM)	1 vial/30 µl	-20°C
700001	DMSO Assay Reagent	1 vial/1 ml	4°C
700002	ADHP Assay Reagent	2 vials	-20°C
400017	96-Well Solid Plate (black)	2 plates	RT
400023	Foil Plate Cover	2 covers	RT
401031	NBD Standard	1 vial/100 µl	-20°C
700023	Resorufin Standard	1 vial/100 µl	-20°C

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

This kit may not perform as described if any reagent or procedure is replaced or modified.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed in the **Materials Supplied** section on page 3 and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader with the ability to kinetically measure fluorescence with an excitation wavelength of 495-505 nm (λ_{max}: 500 nm) and an emission wavelength of 540-560 nm (λ_{max}: 550 nm) for the chlorination assay and an excitation wavelength of 530-540 nm and an emission wavelength of 570-610 nm (λ_{max}: 590 nm) for the peroxidation assay
2. Adjustable pipettes; multichannel or repeating pipettor recommended

INTRODUCTION

Background

Myeloperoxidase (MPO) is a heme-containing enzyme and the most abundant protein in polymorphonuclear leukocytes (PMNs).[1] It is composed of two subunits linked by a disulfide bridge with each subunit containing a light and heavy polypeptide chain. MPO is stored in azurophilic granules of PMNs and is released from activated or necrotic PMNs, after which it can bind to and modify acidic serum proteins, as well as recruit additional PMNs.[1] It can oxidize a variety of substrates and catalyzes the formation of highly reactive (pseudo)hypohalous acids and radicals, including hypochlorous acid (HOCl), using hydrogen peroxide (H_2O_2) for chlorination or peroxidation.[1] The use of H_2O_2 by MPO for either its chlorination or peroxidation activities depends on the relative concentrations of chloride and the reducing substrate.[2] MPO also has roles in PMN apoptosis and antimicrobial defense systems, including neutrophil extracellular trap (NET) formation and NETosis.[1,3,4] It enhances neutrophil elastase-induced chromatin decondensation and produces reactive oxygen species (ROS), which trigger NET formation.[5] MPO-derived oxidants and chlorinated products are enriched in LDL and human atherosclerotic lesions.[6-8] In addition, MPO levels in leukocytes and the blood are elevated in patients with coronary artery disease (CAD), and elevated serum levels of MPO in patients with acute coronary syndromes are considered a risk factor for subsequent cardiovascular events.[7-9] MPO inhibitors improve endothelial function in mouse models of vascular inflammation and atherosclerosis and reduce the severity of cigarette smoke-induced lung damage in a guinea pig model of chronic obstructive pulmonary disease (COPD).[10,11] Development of novel MPO inhibitors has the potential to improve the treatment of conditions caused by oxidative stress.

About This Assay

Cayman's MPO Inhibitor Screening Assay Kit provides convenient fluorometric methods for screening inhibitors to both the chlorination and peroxidation activities of MPO. The chlorination assay utilizes a non-fluorescent thiomorpholine-based probe (NBD-TM), which is selectively cleaved by hypochlorite (-OCl) to yield the highly fluorescent compound, NBD.[12] Fluorescence intensity is analyzed using an excitation wavelength of 500 nm and an emision wavelength of 550 nm. The peroxidation assay utilizes the peroxidase component of MPO, where a single two-electron oxidation of native enzyme (MPO) to compound I (MPO-I) is followed by two successive one-electron reductions back to native enzyme by compound II (MPO-II).[8] The reaction between hydrogen peroxide and 10-acetyl-3,7dihydroxyphenoxazine (ADHP) produces the highly fluorescent compound resorufin. Resorufin fluorescence is analyzed using an excitation wavelength of 530 nm and an emission wavelength of 590 nm. The assay schemes are outlined in Figure 1, below.

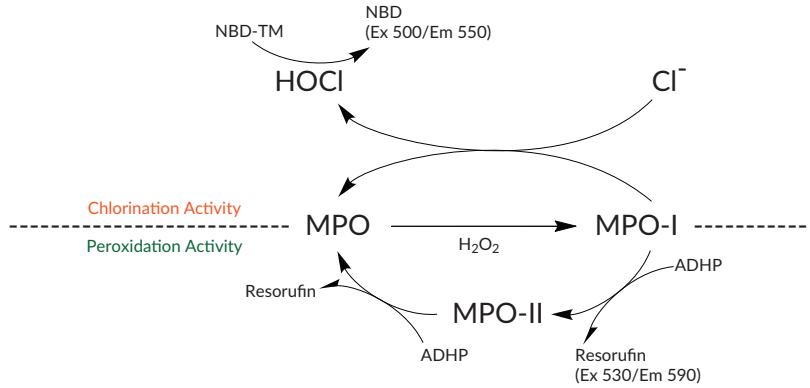


Figure 1. Assay scheme

PRE-ASSAY PREPARATION

Sample Preparation

Test Compounds

Test compounds can be dissolved in DMSO, methanol, or ethanol. Dimethyl formamide (DMF) is not compatible with this assay. Additional solvents have not been tested. The concentrated compound stock solutions must be further diluted in MPO Assay Buffer (1X) to a concentration 11X the final assay (*i.e.* in-well) concentration. To minimize potential interference by solvents in the assay, refer to the **Effects of Solvents** section (pages 21 and 22) to determine the maximum solvent concentration tolerated by the assay. Appropriate vehicle control wells containing the same concentration of solvent used for the test compounds must be included in each assay.

Reagent Preparation

1. **MPO Assay Buffer (1X) - (Item No. 700164)**

   This bottle contains 50 ml of MPO Assay Buffer (1X). It is ready to use as supplied.

2. **MPO Enzyme (human) - (Item No. 700166)**

   This vial contains a 40 µl suspension of human PMN MPO Enzyme. MPO enzyme should be thawed on ice and mixed by gently pipetting up and down prior to dilution as the enzyme may settle over time. *Do not vortex.* Dilute the MPO Enzyme 1:1,600 to prepare the MPO enzyme working solution by mixing 5 µl of MPO Enzyme with 7,995 µl of MPO Assay Buffer (1X). This is a sufficient volume to evaluate a full plate. Scale as needed. It is recommended that the enzyme be diluted just prior to performing the assay. The diluted enzyme should be kept on ice where it will be stable for one hour. Any remaining undiluted enzyme can be aliquoted and stored at -20°C, limiting freeze-thaw cycles.

3. **MPO Inhibitor - (Item No. 700167)**

   This vial contains 50 µl of 50 mM 4-aminobenzhydrazide in DMSO, which can be used as an inhibitor control. Mix 10 µl of MPO Inhibitor with 490 µl MPO Assay Buffer (1X) to make a 1 mM working solution. If all of the MPO Inhibitor will not be used at one time, aliquot the undiluted inhibitor and store at -20°C.

4. **MPO Hydrogen Peroxide - (Item No. 700168)**

   This vial contains 100 µl of an 8.8 M solution of hydrogen peroxide. Prior to the assay, mix 10 µl with 990 µl of MPO Assay Buffer (1X) to yield an 88 mM solution. Then, mix 10 µl of the 88 mM solution with 990 µl of MPO Assay Buffer (1X) to prepare a 0.88 mM solution. The 0.88 mM solution will be used to prepare the initiator solutions (see page 16). The diluted hydrogen peroxide solutions will be stable for one hour at room temperature.

Chlorination-Specific Reagent Preparation

1. **MPO Chlorination Detector (NBD-TM) - (Item No. 400823)**

   This vial contains 30 µl of MPO Chlorination Detector (NBD-TM) in DMSO. Prior to preparing the chlorination initiator solution (see page 16), dilute 500-fold by mixing 10 µl of NBD-TM with 4,990 µl of MPO Assay Buffer (1X). Store on ice and do not vortex. This is a sufficient volume to evaluate half a plate. Scale as needed. The diluted MPO Chlorination Detector (NBD-TM) will be stable for one hour when stored on ice. Any remaining undiluted reagent can be aliquoted and stored at -20°C. Limit freeze-thaw cycles to two.

2. **NBD Standard - (Item No. 401031)**

   This vial contains 100 µl of NBD in ethanol, which will be used as a calibrator to optimize the instrument's gain setting when running the chlorination assay. Add 10 µl of NBD Standard to 990 µl of MPO Assay Buffer (1X). Further dilute by adding 50 µl to 450 µl of MPO Assay Buffer (1X) to yield the NBD calibrator solution. This will be stable at room temperature for 2 hours.

Peroxidation-Specific Reagent Preparation

1. **DMSO Assay Reagent - (Item No. 700001)**

   This reagent is ready to use to prepare the MPO Peroxidation Substrate (see below).

2. **MPO Peroxidation Substrate**

   Immediately prior to preparing the peroxidation initiator solution (see page 16), add 120 µl of DMSO Assay Reagent to one vial of ADHP Assay Reagent (Item No. 700002) and vortex until dissolved. Then, add 470 µl of MPO Assay Buffer (1X) for a final MPO Peroxidation Substrate concentration of 1 mM. This is a sufficient volume to assay half a plate. Prepare the second vial if running a full plate. The MPO Peroxidation Substrate will be stable for 30 minutes at room temperature.

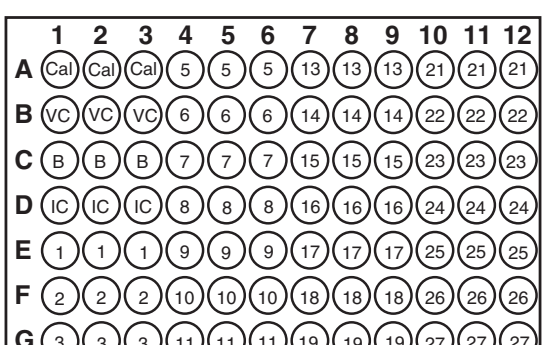
3. **Resorufin Standard - (Item 700023)**

   This vial contains resorufin, which will be used as a calibrator to optimize the instrument's gain setting when running the peroxidation assay. Add 10 µl of Resorufin Standard to 790 µl of MPO Assay Buffer (1X). Further dilute by adding 10 µl to 790 µl of MPO Assay Buffer (1X) to yield the resorufin calibrator solution. This will be stable for two hours at room temperature.

ASSAY PROTOCOL

Plate Set Up

There are two plates included in this kit, one for the chlorination assay and one for the peroxidation assay. Chlorination and peroxidation assays cannot be measured simultaneously on the same plate. There is no specific pattern for using the wells on the plate. However, it is recommended to have each plate contain three wells designated as vehicle control, three wells designated as background, and three wells designated as calibrator wells. It is suggested that each test compound, including the inhibitor control, be assayed in triplicate. It is suggested that the contents of each well may be recorded on the template sheet provided on page 27. A typical layout of samples to be measured in triplicate is shown in Figure 2, on page 13.



Cal = Calibrator Wells
VC = Vehicle Control Wells
B = Background Wells
IC = Inhibitor Control Wells
1-28 = Test Compound Wells

Figure 2. Sample plate format

Pipetting Hints

- It is recommended that a multichannel pipette should be used to deliver reagents to the wells. This saves time and helps maintain more precise incubation times.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume of the assay is 110 µl in all the wells.
- All reagents should be prepared as described above. The MPO enzyme and the diluted MPO Chlorination Detector (NBD-TM) should be kept on ice and all other reagents should be kept at room temperature before beginning the assay.
- It is not necessary to use all the wells on the plate at one time.
- It is recommended that samples be assayed in triplicate, but it is at the user's discretion to do so.
- Both assays are performed at room temperature.
- Chlorination and peroxidation assays cannot be measured simultaneously on the same plate.
- Monitor the chlorination fluorescence with an excitation wavelength of 500 nm and an emission wavelength of 550 nm.
- Monitor the peroxidation fluorescence with an excitation wavelength of 530 nm and an emission wavelength of 590 nm.

Performing the Assays

NOTES: The chlorination and peroxidation assays cannot be run simultaneously on the same plate. For best performance, the initiator solutions (step 2, Table 2 on page 16) should be prepared just prior to initiating the reaction. Preset the instrument to optimize the gain to the calibrator wells. Add the initiator solution to the wells next to the plate reader and begin fluorescence readings immediately.

1. Add 110 µl of NBD or resorufin calibrator solution to the designated calibrator wells.
2. Read the plate to determine the optimal *gain* settings for the chlorination assay (ex/em: 500/550) nm or the peroxidation assay (ex/em: 530/590 nm). The optimal *gain* setting will then be used when assaying the test compounds.
3. Add the appropriate amount of prepared reagents to the designated wells according to Table 1, below.

Reagents	Vehicle Control Wells	Inhibitor Control Wells	Test Compound Wells	Background Wells
MPO Assay Buffer (1X)	--	--	--	50 µl
MPO Enzyme Working Solution	50 µl	50 µl	50 µl	--
Vehicle Solution	10 µl	--	--	10 µl
Diluted MPO Inhibitor	--	10 µl	--	--
Test Compound	--	--	10 µl	--

Table 1. Pipetting summary

4. Prepare the initiator solutions according to Table 2, below, and use within 15 minutes. Scale as needed. The chlorination initiator solution will turn yellow.

Reagents	Chlorination Initiator Solution (50 wells)	Peroxidation Initiator Solution (50 wells)
MPO Assay Buffer (1X)	3,590 µl	3,590 µl
Diluted MPO Chlorination Detector (NBD-TM)	400 µl	--
MPO Peroxidation Substrate	--	400 µl
Hydrogen Peroxide (0.88 mM)	10 µl	10 µl
Final Volume	4,000 µl	4,000 µl

Table 2. Initiator solutions preparation

5. Initiate the reactions by adding 50 µl of initiator solution to all the wells except the calibrator wells. Mixing the contents is not necessary.
6. Read kinetically by measuring the fluorescence every 30 seconds at room temperature for 10 minutes. For the chlorination assay, read at an excitation wavelength of 500 nm and an emission wavelength of 550 nm. For the peroxidation assay, read at an excitation wavelength of 530 nm and an emission wavelength of 590 nm.

ANALYSIS

Calculations

1. Determine the change in fluorescence (ΔF) per minute:
   a. Plot the fluorescence values as a function of time and perform a linear regression to obtain the slope (rate) of the linear portion of the curve (for example data, see Figure 3 on page 19). Use the absolute value in subsequent steps.

   OR

   b. Select two points on the linear portion of the curve and determine the change in fluorescence over time using the following equation:

$$\Delta F/min = \frac{|\ Fluorescence\ (time\ 2) - Fluorescence\ (time\ 1)\ |}{time\ 2\ (min) - time\ 1\ (min.)}$$

2. Determine the background-corrected values by subtracting the average rate of the background wells from the vehicle control and test compound average rates.
3. Use the background-corrected values to determine the percent inhibition or percent activity for each test compound using one of the following equations:

$$\%\ inhibition = \left[1 - \frac{corrected\ value\ of\ test\ compound}{corrected\ value\ of\ vehicle}\right] \times 100$$

$$\%\ activity = \frac{corrected\ value\ of\ test\ compound}{corrected\ value\ of\ vehicle} \times 100$$

4. Graph the percent inhibition or percent activity as a function of test compound concentration to determine the IC_{50} value (the concentration at which there is 50% inhibition) of the inhibitor. Inhibition of MPO by the inhibitor control using the chlorination and peroxidation assays are shown in Figure 3 (see page 19).

Z´ Factor

Z´ factor is a term used to describe the robustness of an assay, which is calculated using the equation below.[13]

$$Z' = 1 - \frac{3\sigma_{c+} + 3\sigma_{c-}}{|\mu_{c+} - \mu_{c-}|}$$

Where σ: Standard deviation
μ: Mean
c+: Positive control
c-: Negative control

The theoretical upper limit for the Z' factor is 1.0. A robust assay has a Z' factor >0.5. The Z' factors for Cayman's Myeloperoxidase Chlorination and Peroxidation Inhibitor Screening Assay Kit for the chlorination and peroxidation assays were determined to be 0.79 and 0.89, respectively.

Performance Characteristics

Sample Data

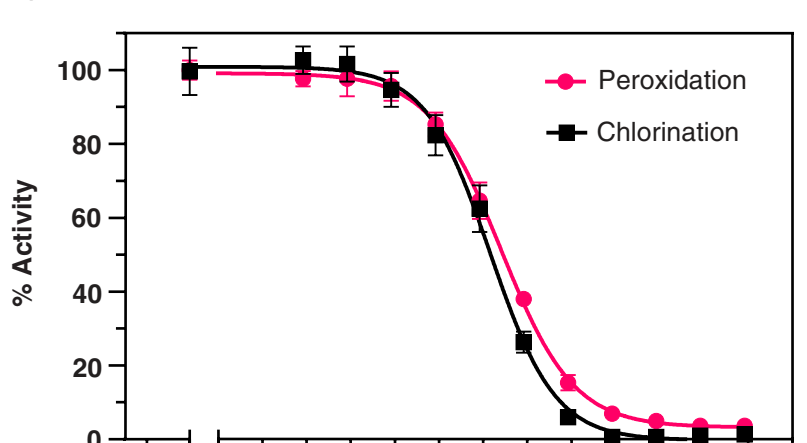


Figure 3. Inhibition of human PMN MPO by MPO inhibitor. Data are plotted as the mean of triplicate measurements ± the standard deviation. The vehicle control (Veh.) represents 100% activity. The IC_{50} values of MPO Inhibitor are 12 and 16 nM for the chlorination assay and peroxidation assay, respectively.

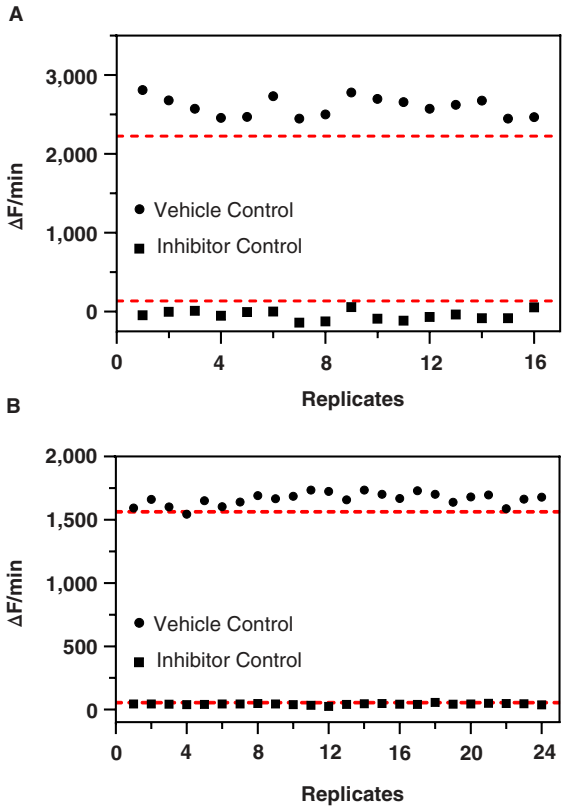


Figure 4. Typical performance data for the MPO Chlorination and Peroxidation Inhibitor Screening Assay Kit. Data are shown from 16 and 24 replicates for the chlorination (A) and peroxidation (B) assays, respectively. The inhibitor control was prepared as described in the kit booklet. The calculated Z' factor for the experiments shown above were 0.79 and 0.89 for the chlorination and peroxidation assays, respectively. The dashed/dotted lines correspond to three standard deviations from the mean for each control value.

Effects of Solvents

A titration of organic solvents showed that signal can change with increasing solvent concentration, so the proper vehicle control should be included in the assay.

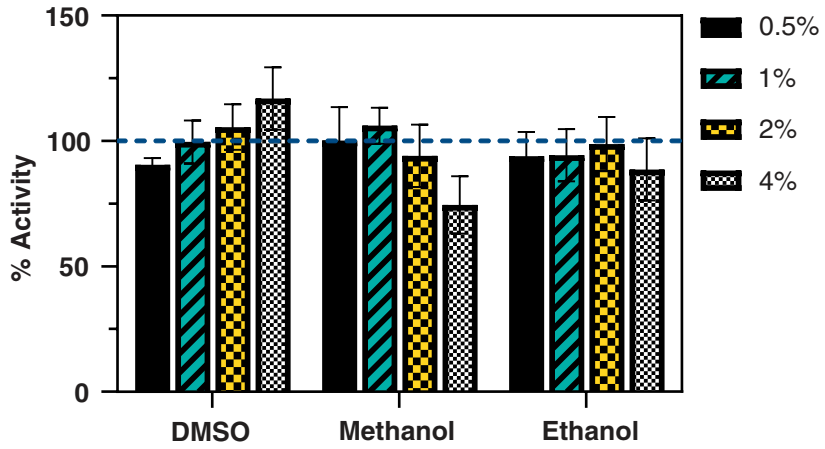


Figure 5. The effect of solvent on the readout of MPO chlorination activity. The data are shown as the mean ± standard deviation for triplicate reactions containing the indicated concentration of solvents. The dashed line represents the no solvent control.

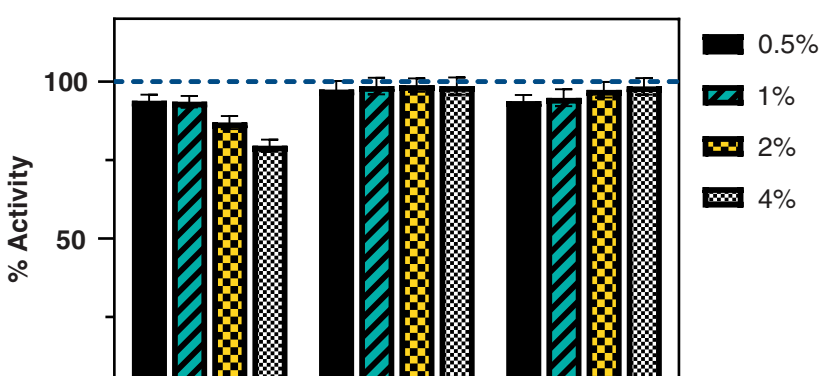


Figure 6. The effect of solvent on the readout of MPO peroxidation activity. The data are shown as the mean ± standard deviation for triplicate reactions containing the indicated concentration of solvents. The dashed line represents the no solvent control.

RESOURCES

Item Number	Reagent	Procedure
700164	MPO Assay Buffer (1X)	Ready to use as supplied
700166	MPO Enzyme (human)	Dilute 1:1,600 with Assay Buffer (1X); keep on ice
700167	MPO Inhibitor	Dilute 1:50 with Assay Buffer (1X)
700168	MPO Hydrogen Peroxide	Dilute 1:100 with Assay Buffer (1X); further dilute 1:100 to make a 0.88 mM solution
400823	MPO Chlorination Detector (NBD-TM)	Dilute 1:500 with Assay Buffer (1X)
700002	ADHP Assay Reagent	To make the *MPO Peroxidation Substrate*, reconstitute with 120 µl DMSO (Item No. 700001) and vortex; then, add 470 µl of Assay Buffer 1X; use within 30 min
401031	NBD Standard	Dilute 1,000-fold with Assay Buffer (1X)
700023	Resorufin Standard	Dilute 6,400-fold with Assay Buffer (1X)

Table 3. Assay reagent preparation summary

Reagent	Chlorination (50 wells)	Peroxidation (50 wells)
MPO Assay Buffer (1X)	3,590 µl	3,590 µl
Diluted MPO Chlorination Detector (NBD-TM)	400 µl	--
Reconstituted MPO Peroxidation Substrate	--	400 µl
Hydrogen Peroxide (0.88 mM)	10 µl	10 µl
Final Volume	4,000 µl	4,000 µl

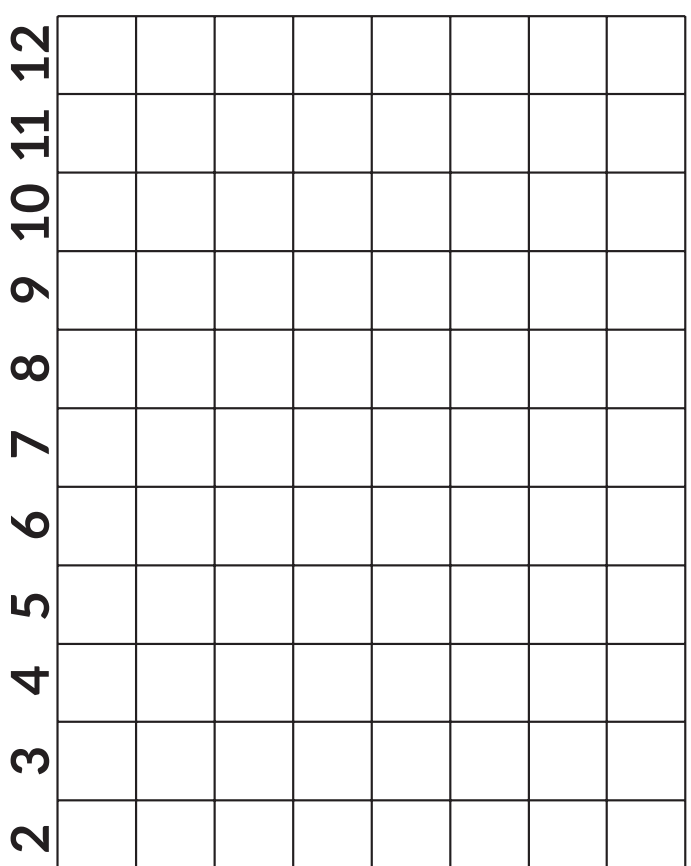
Table 4. Initiator solutions preparation

Procedure	Background Wells	Vehicle Control Wells	Inhibitor Control Wells	Test Compound Wells	Calibrator Wells
Add MPO Assay Buffer (1X)	50 µl	--		--	--
Add diluted-MPO Enzyme	--	50 µl	50 µl	50 µl	--
Add vehicle	10 µl	10 µl	--	--	--
Add diluted MPO Inhibitor	--	--	10 µl	--	--
Add test compound (11X)	--	--	--	10 µl	--
Add appropriate calibrator solution	--	--	--	--	110 µl
Initiate with appropriate initiator solution	50 µl				--
Read	Read fluorescence once every 30 seconds for 10 minutes at the following emission and excitation wavelengths: Chlorination: Ex = 500 nm; Em = 550 nm Peroxidation: Ex = 530 nm; Em = 590 nm				Use calibrator wells to determine optimal *gain* setting

Table 5. Assay summary

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of replicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
The ΔF/min of the test compound wells is similar to the ΔF/min of the background wells	The test compound is a potent inhibitor of the enzyme	Reduce the concentration of the inhibitor and re-assay
The plate reader exhibited 'MAX' values for the wells	The *gain* setting is too high	Reduce the *gain* and re-read
No inhibition was seen with test compound	A. The inhibitor concentration is not high enough B. The compound is not an inhibitor of the enzyme	Increase the inhibitor concentration and re-assay
The ΔF/min of the vehicle control is similar to the ΔF/min of the background wells	A. The linear portion may have been missed B. No enzyme was added or the enzyme is no longer active	A. Prepare all reagents correctly and use within the specified time frame. Initiate the reactions next to the plate reader and do not delay measuring fluorescence B. If the enzyme was added, be sure to follow proper handling and storage instructions



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NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.